CompCytogen 16(3): 161–172 (2022)
doi: 10.3897/compcytogen.v16.i3.90336
https://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Karyotype and COI gene sequences of *Chironomus melanotus* Keyl, 1961 from the Yaroslavl region, Russia, and the difficulties with its identification using GenBank and BOLD systems

Viktor V. Bolshakov[1], Ekaterina A. Movergoz[1]

1 *Papanin Institute for Biology of Inland Waters Russian Academy of Sciences, Yaroslavl reg., Nekouz prov., Borok, 152742, Russia*

Corresponding author: Viktor V. Bolshakov (victorb@ibiw.ru)

Academic editor: Paraskeva Michailova | Received 13 July 2022 | Accepted 28 August 2022 | Published 14 September 2022

https://zoobank.org/5A6F7311-F8F5-4127-82FE-8396259D0F60

Citation: Bolshakov VV, Movergoz EA (2022) Karyotype and COI gene sequences of *Chironomus melanotus* Keyl, 1961 from the Yaroslavl region, Russia, and the difficulties with its identification using GenBank and BOLD systems. Comparative Cytogenetics 16(3): 161–172. https://doi.org/10.3897/compcytogen.v16.i3.90336

Abstract

Karyotype and *COI* gene sequences of *Chironomus melanotus* Keyl, 1961 from the Yaroslavl region (Russia) were analyzed. A low level of chromosomal polymorphism has been confirmed, eventually eight banding sequences were found: melA1, melB1, melC1, melD1, melE1, melF1, and melG1; only melD2 was found in two larvae from the Sunoga river. Analysis of phylogenetic tree and estimated genetic distances has shown not all *COI* gene sequences of *Ch. melanotus* in GenBank and BOLD to belong to this species. The lower distance of 0.4% was observed between two sequences from the Yaroslavl region and Finland, apparently these are true *Ch. melanotus* sequences. The distances between true *Ch. melanotus* and other sequences from Finland were 9.5% and 12.4%, and from Sweden it was 11%. The average genetic distance between studied sequences of 9.1% is out of the range of the 3% threshold previously determined for chironomids. According to our estimates, there are two sequences with a distance of 2.9% that may belong to *Ch. annularius* Meigen, 1818, and one sequence with a genetic distance of 2.1%, may belonging to *Ch. cingulatus* Meigen, 1830, which has been confirmed karyologically. Another two sequences form a separate cluster. We suggest that they either belong to a known species, but are not present in the databases, or belong to a distinct, undescribed species.

Keywords

Chironomidae, *Chironomus melanotus*, *COI*, dark taxa, DNA-barcode, karyotype

Copyright *Viktor V. Bolshakov & Ekaterina A. Movergoz*. This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Introduction

Chironomus melanotus Keyl, 1961 is one of the most widespread and well-known species. It does not belong to any sibling species group (Kiknadze et al. 2010). The first finding and description was in Germany (Keyl 1961, 1962; Degelmann et al. 1979). *Ch. melanotus* is in demand in classical hydrobiology (Fjellheim, Raddum 1996) and in toxicology (Grebenjuk 1994; Grebenjuk and Tomilina 2014). The main problem in the investigation of *Chironomus* Meigen, 1803 is the difficulties with the species identification by larval morphology. Due to the presence of the giant chromosomes in the salivary gland of *Chironomus* larvae, it is more convenient to identify cytogenetically (Kiknadze et al. 1991, 2016). The karyotype of *Ch. melanotus* was described by Keyl (1961) as a "cytospecies" that belongs to "thummi" cytocomplex and mapped chromosomal arms A and F (Keyl, 1962). It has been shown that the level of polymorphism in *Ch. melanotus* is very low (Wülker 1973; Kiknadze et al. 1991; Jabłońska-Barna et al. 2013). Only in polluted water bodies a high spectrum of somatic rearrangements and a case of trisomy were observed (Jabłońska-Barna et al. 2013). Finally, due to the development of new techniques in molecular biology, for species identification/delimitation the fast and cost-effective technology DNA barcoding is commonly used and for massive analysis in biomonitoring metabarcoding is used. In recent years, many works on this theme have been published. This is a barcoding of invertebrates, including chironomids from Canada (Hebert et al. 2016), Germany (Morinière et al. 2019), Finland (Roslin et al. 2022), South Korea (Kang et al. 2022), Montenegro/Albania (Gadawski et al. 2022) and others. The disadvantage of this approach is the presence in the databases of genetic information (GenBank and BOLD) from unidentified or incorrectly identified specimens, so-called "dark taxa" (Morinière et al. 2019). The next problem is the understanding that a sequence divergence threshold is not suitable for all *Chironomus* species and depends on intraspecific and interspecific sequence divergences. Interspecific - varied for *COI* gene sequences in most cases from 9 to 20% and in rare cases from 1 to 4% (Proulx et al. 2013). Due to the fact that we cannot fully estimate intra- and inter-specific sequence divergences, here we will use the average value of this parameter – 3% (Ekrem et al. 2007; Proulx et al. 2013; Kondo et al. 2016).

We could not find any studies of *Ch. melanotus* involving approaches of morphology, cytogenetics and DNA barcoding published in one article. In the GenBank and BOLD databases were found five and one *COI* gene sequences, respectively. These sequences were obtained from individuals collected in Finland and Sweden, and deposited during the preparation of this paper (Roslin et al. 2022). Preliminary examination has shown that not all of these sequences belong to *Ch. melanotus*.

The present study aims to calculate and compare the genetic distances between *COI* gene sequences of *Ch. melanotus* from Yaroslavl region identified by morphology and cytogenetics and the sequences obtained from GenBank and BOLD of *Ch. melanotus* from different populations identified by morphology or molecular-genetics (barcode), and additional sequences from GenBank and BOLD of several *Chironomus* identified by cytogenetics.

Materials and methods

Fourth instar larvae of *Ch. melanotus* were collected from a few places in the Yaroslavl region, Russia. Thirty-one larvae were found in a puddle on the Shumarovka river shore (58°02'25.5"N, 38°15'33.2"E) in October 2018. The depth is 0.5 m, and the bottom is black silt. Seven larvae were collected in the Sunoga river (58°03'20.3"N, 38°14'04.2"E) in August 2018. The depth is 0.1 – 0.2 m, and the bottom is gray silt with sand. Four larvae were collected in a small stream (brook) in the shore zone of the Kotorosl' river (57°22'41.6"N, 39°50'08.5"E) in June 2016. The depth is 0.5 m, and the bottom is black silt and rotting wood.

The age was determined by the standard method (Ilyinskaya, 1983). All larvae were taken for karyotype analysis using the ethanol-orcein technique (Dyomin 1989). A Micromed-6C (LOMO, St. Petersburg) light microscope equipped with a standard (kit) oil objective ×100 and a camera ToupCam5.1 (China) were used for microscopy analysis. To identify chromosome banding sequences, the cytomaps by Kiknadze et al. (1991, 2016), Keyl (1961, 1962), Hirvenoja and Michailova (1991) were used. Preparations of *Ch. melanotus* have been deposited at IBIW RAS.

One larva from a small stream (brook) in the shore zone of Kotorosl river studied karyologically was taken for the total DNA extraction using "M-sorb-OOM" (Sintol, Moscow) kit with magnet particles according to manufacturer's protocol. For amplification of *COI* (cytochrome oxidase subunit I) we used primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA -3') (Eurogen, Moscow) (Folmer et al. 1994). Amplification reaction was carried out in 25 μl reaction mixture (1x buffer, 1.5 μM MgCl2, 0.5 mM of each primer, 0.2 μM dNTP of each nucleotide, 17.55 μL deionized water, 1 μL template DNA, 1 unit Taq-polymerase (Evrogen, Moscow). PCR was performed at 94 °C (3 min), followed by 30 cycles at 94 °C (15 s), 50 °C (45 s), 72 °C (60 s) and a final extension at 72 °C (8 min). PCR products were visualized on 1% agarose gels and later purified by ethanol and ammonium acetate (3 M). Both strands were sequenced on an Applied Biosystems 3500 DNA sequencer (Thermo Scientific, USA) following the manufacturer's instructions.

For alignment of *COI* nucleotide sequences, we used MUSCLE in the MEGA6 software (Tamura et al. 2013). The MEGA6 was used to calculate pairwise genetic distances (p-distance) with codon position preferences: 1st, 2nd, 3rd and noncoding sites. The Bayesian analysis was performed using the program MrBayes v.3.2.6 (Ronquist and Huelsenbeck 2003; Ronquist et al. 2012) with settings suggested by Karmokov (2019; Bolshakov, Prokin 2021), for 1 000 000 iterations and 1000 iterations of burn-in, nst = 6 (GTR + I + G). The phylogenetic trees resulting in Bayesian inference analyses were visualized and edited using FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

In addition, thirty-four *COI* gene sequences of the genus *Chironomus* from "GenBank" and "Barcode of Life Data Systems" (BOLD) were analyzed. Accession numbers of used sequences in GenBank and BOLD: *Ch. acutiventris* Wülker et al., 1983 (AF192200.1), *Ch. annularius* Meigen, 1818 (AF192189.1), *Ch. anthracinus*

Zetterstedt, 1860 (KF278222), *Ch. balatonicus* Devai et al., 1983 (JN016826.1), *Ch. bernensis* Wülker et Klötzli, 1973 (AF192188.1), *Ch. borokensis* Kerkis et al., 1988 (AB740261), *Ch. cingulatus* Meigen, 1830 (AF192191.1), *Ch. commutatus* Keyl, 1960 (AF192187.1), *Ch. curabilis* et al., 1990 (JN016810.1), *Ch. dilutus* et al., 1999 (KF278335.1), *Ch. entis* Shobanov, 1989 (KM571024.1), *Ch. heterodentatus* Konstantinov, 1956 (AF192199.1), *Ch. heteropilicornis* Wülker, 1996 (MK795770.1), *Ch. maturus* Johannsen, 1908 (DQ648204.1), *Ch. melanotus* (MZ659620, MZ657748, MZ658877, MZ657558, MZ658420, BSCHI737-17), *Ch. nipponensis* Tokunaga, 1940 (DQ648206), *Ch. novosibiricus* Kiknadze et al., 1993 (AF192197.1), *Ch. nuditarsis* Keyl, 1961 (KY225345.1), *Ch. obtusidens* Goetghebuer, 1921 (CHMNO207-15*), *Ch. piger* Strenzke, 1959 (AF192202.1), *Ch. pilicornis* Fabricius, 1787 (HM860166.1), *Ch. plumosus* Linnaeus, 1758 (KF278217.1), *Ch. riparius* Meigen, 1804 (KR756187.1), *Ch. sokolovae* Istomina et al., 1999 (MW471100), *Ch. sororius* Wulker, 1973 (MZ324811), *Ch. tentans* Fabricius, 1805 (AF110157.1), *Ch. tuvanicus* Kiknadze et al., 1993 (AF192196.1), *Ch. usenicus* Loginova et Belyanina, 1994 (JN016820.1), *Ch. whitseli* Sublette et Sublette, 1974 (KR683438.1). The *COI* sequence of *Drosophila melanogaster* Meigen, 1830 (HQ551913) was used as outgroup in phylogenetic analysis.

Results and discussion

The karyotype of *Chironomus melanotus* Keyl, 1961 from the Yaroslavl region, Russia

The species has a 2n = 8 set of chromosomes. By the chromosome arm combination – AB, CD, EF and G, the species belongs to *Chironomus* "thummi" cytocomplex. The chromosomes AB and CD are metacentric, EF is submetacentric, and G is telocentric. The nucleus and Balbiani ring were found in arm G. The peculiarity of the karyotype of *Ch. melanotus* is a heterochromatinized centromeric region that forms an unstable chromocenter (Fig. 1), also observed only in *Ch. cucini* Webb, 1969, *Ch. pilicornis*, *Ch. athalassicus* Cannings 1975, *Ch. magnus* White et Ramsey, 2015 and *Ch. hyperboreus* Staeger, 1845 (Wülker and Butler 1983; Int Panis et al. 1994; Kiknadze and Istomina 2000; Wülker and Martin 2000; Kiknadze et al. 2010).

We found two zygotic combinations: melA1.1. B1.1. C1.1. D1.1. E1.1. F1.1. G1.1, and melA1.1. B1.1. C1.1. D1.2. E1.1. F1.1. G1.1, which was found only in two larvae from the Sunoga river.

All eight banding sequences coincide with banding sequences in Keyl et al. (1961, 1962), Hirvenoja and Michailova (1991) and Kiknadze et al. (1991, 2016).

Arm A. One banding sequence: melA1 1a-2c 10a-12c 3i-2h 4d-9e 2g-d 4c-a 13a-19f C.

Arm B. One banding sequence: melB1 28-27-26-25-24-23-22-21-20-19 C (mapped according to Hirvenoja, Michailova 1991). Different from *Ch. plumosus* by four inversion steps.

Arm C. One banding sequence: melC1 1a-2e 15c-e 11c-8a 6b-2f 15b-11d 6gh 17a-16a 7d-a 6f-c 17b-22g C.

Arm D. Two banding sequences: melD1 1a-3g 11a-13a 16a-e 8a-10a 13b-15e 10e-b 4a-7g 17a-24g C in heterozygous state with melD2 1a-3g 11a-13a 16a-e 8a-9e 7g-4a 10b-e 15e-13b 10a 17a-24g C.

Arm E. One banding sequence: melE1 1a-3e 5a-10b 4h-3f 10c-13g C.

Arm F. One banding sequence: melF1 1a-8f 9a-11i 12a-15i 16a-23f C.

Arm G. One banding sequence: melG1. Not mapped.

The level of polymorphism in *Ch. melanotus* is known to be very low (Kiknadze et al. 1991; Jabłońska-Barna et al. 2013). At the moment, we know of two alternative banding sequences, melB2 and melD2, and the sequence melD2 is more typical for Western Siberia populations (Kiknadze et al. 2016) and a Finnish population (Hirvenoja et Michailova 1991). We found melD2 in a heterozygous state in two larvae from the Sunoga river. Any deviations in the karyotype characteristics like a trisomy, rearrangements, insertions and deletions, more typical for polluted water bodies (Jabłońska-Barna et al. 2013) were not found.

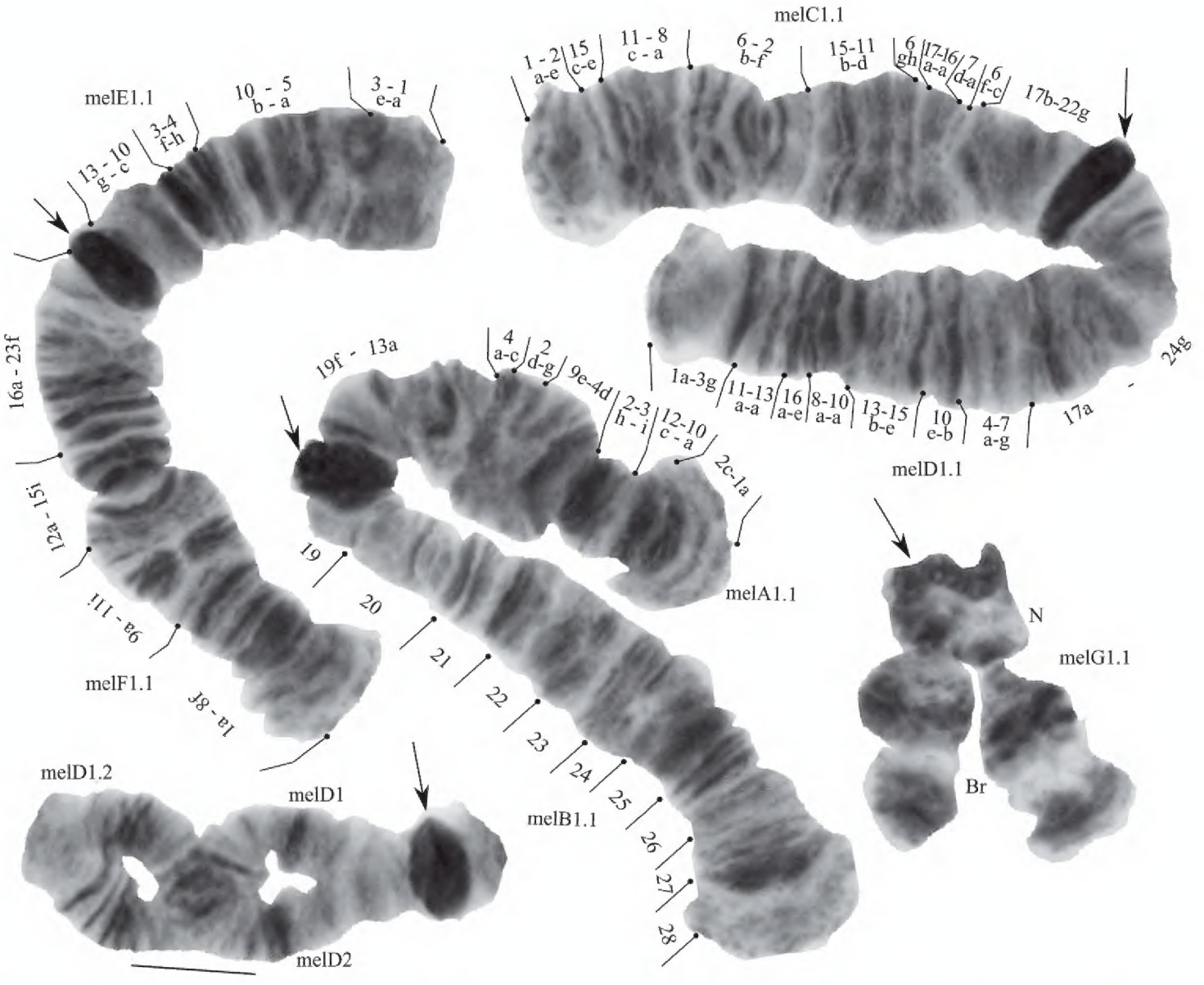

Figure 1. Karyotype of *Chironomus melanotus* from the Sunoga river, Yaroslavl, Russia. Arrows indicate centromeric bands, melA1, melB1 and etc. – genotypic combinations of banding sequences in chromosome arms, BR – Balbiani rings, N – nucleous.

DNA-barcoding and phylogenetic analysis

The obtained *COI* gene sequence for *Ch. melanotus* from the Yaroslavl region was deposited in the GenBank with accession number OL546775; the length of the sequence is 658 bp (percentage of nucleotides A: 25; T: 38; G: 17; C: 19).

More interesting was the analysis of *COI* gene sequences. As was said previously, for the species name *Ch. melanotus* in the databases match six sequences of the *COI* gene from Finland (MZ659620, MZ657748, MZ658877, MZ657558, MZ658420) identified by molecular-genetics and Sweden (BSCHI737-17) identified by imago characters, and the average genetic distance between them of 9.1% is out of the range of 3% distances previously determined for chironomids (Ekrem et al. 2007; Proulx et al. 2013; Kondo et al. 2016). Low chromosomal variability of *Ch. melanotus* does not allow us to talk about a high level of genetic diversity. We can conclude not all sequences belong to *Ch. melanotus* species (Table 1). According to our estimation, the lower distance, about 0.4%, was between *Ch. melanotus* (MZ659620) from Finland and Yaroslavl reg. (OL546775). The distance between sequences of *Ch. melanotus* (OL546775) from Yaroslavl reg. and sequences from Finland (MZ657748, MZ658877) - 9.5%, (MZ657558, MZ658420) - 12.4%, and Sweden (BSCHI737-17) - 11%. These values are greater than those between sequences from the Yaroslavl reg. (OL546775) and *Ch. anthracinus* (KF278222), identified karyologically (Proulx et al. 2013), with a distance of 4%. This still doesn't mean these species are really closely related, the analysis of one short segment of the *COI* gene is not enough to make such conclusions (DeSalle et al. 2005). However, a high similarity of their karyotypes has been noted, up to identity of some banding sequences (Keyl 1962; Kiknadze et al. 1991).

The distance between the two similar sequences (MZ657558 and MZ658420) from Finland and *Ch. annularius* (AF192189.1) confirmed karyologically (Guryev et al. 2001) was 2.9%; between sequences (BSCHI737-17) from Sweden and *Ch. cingulatus* (AF192191.1) confirmed karyologically (Guryev et al. 2001) - 2.1%, *Ch. nipponensis* (DQ648206) - 4.2%, identified by morphology and molecular-genetics (Kondo et al. 2016). Two similar sequences are particularly interesting (MZ657748 and MZ658877), the distances between of them and all the analyzed sequences varied from 6.5 to 10.5%, and the average was 12%. Unfortunately, we didn't find any matches in GenBank and BOLD.

On the phylogenetic tree constructed by Bayesian inference (Fig. 2), we see the same situation as with the genetic distances. The sequence of *Ch. melanotus* (OL546775) from Yaroslavl reg., and *Ch. melanotus* (MZ659620) from Finland combined into one cluster, while the other sequences spread out into different branches. Two similar sequences (MZ657558 and MZ658420) from Finland and *Ch. annularius* (AF192189.1) combined in one cluster, with a support value of 1.0. The sequence (BSCHI737-17) from Sweden and *Ch. cingulatus* (AF192191.1) formed another cluster, with a support value of 1.0. Two similar sequences from Finland (MZ657748 and MZ658877) have formed a separate cluster, without including any other specimens.

Table 1. Pairwise genetic distances (p-distances, %) between *COI* gene sequences of *Ch. melanotus* and closest sequences of *Chironomus* from GenBank and BOLD.

	Ch. melanotus OL546775 Yaroslavl, RUS	*Ch. melanotus* MZ659620 Finland	*Ch. melanotus* MZ657748 Finland	*Ch. melanotus* MZ658877 Finland	*Ch. melanotus* BSCHI737-17 Sweden	*Ch. melanotus* MZ657558 Finland	*Ch. melanotus* MZ658420 Finland	*Ch. anthracinus* KF278222	*Ch. annularius* AF192189.1	*Ch. cingulatus* AF192191.1
Ch. melanotus MZ659620 Finland	0,4									
Ch. melanotus MZ657748 Finland	9,5	9,7								
Ch. melanotus MZ658877 Finland	9,5	9,7	0,0							
Ch. melanotus BSCHI737-17 Sweden	11,0	11,4	7,0	7,0						
Ch. melanotus MZ657558 Finland	12,4	12,6	10,7	10,7	11,2					
Ch. melanotus MZ658420 Finland	12,4	12,6	10,7	10,7	11,2	0,4				
Ch. anthracinus KF278222	4,0	3,6	10,5	10,5	11,6	12,0	12,4			
Ch. annularius AF192189.1	12,6	12,8	9,7	9,7	10,1	2,9	2,9	12,6		
Ch. cingulatus AF192191.1	10,9	11,2	7,2	7,2	2,1	11,0	11,0	11,8	10,1	
Ch. nipponensis DQ648206	10,3	10,7	6,5	6,5	4,2	11,4	11,4	11,2	9,9	4,8

All the obtained data show that several species are hidden in GenBank and BOLD under the name "*Chironomus melanotus*". First, there is a true *Ch. melanotus* cluster (Fig. 2) (MZ659620 and OL546775) the reliability of which is confirmed by karyotype analysis. Probably, two similar sequences (MZ657558 and MZ658420) belong to *Ch. annularius* (AF192189.1), and the genetic distance of 2.9% is very close to 3% accepted interspecific threshold (Ekrem et al. 2007; Proulx et al. 2013; Kondo et al. 2016), but does not exceed it. Another sequence (BSCHI737-17), with a genetic distance of 2.1%, likely belongs to *Ch. cingulatus* (AF192191.1).

Two similar sequences (MZ657748 and MZ658877) need special attention. The samples of *Ch. melanotus* from Finland were investigated during the project of FinBOL (Finnish Barcode Of Life), in the framework of which the authors tested the system FinPROTAX (Probabilistic Taxonomic Assignment Tool) (Roslin et al. 2022). As the authors report, the accuracy of taxonomic assignments at the level of species reached 88.5% (Roslin et al. 2022). Such precision is still insufficient, especially in a group rich in sibling species. This approach does not consider estimate intra- and inter-specific sequence divergences. For the *COI* gene, the estimated interspecific sequence divergences in most cases varied from 9 to 20%, but in a few cases with well-identified by cytogenetics species, this

parameter varied from 1 to 4%, which overlapped between intraspecific and interspecific sequence divergences (Proulx et al. 2013). According to Morinière et al. (2019), the database of genetic information contains about 65% of sequences without species-level assignment, so-called "dark taxa" of all Chironomidae recorded from Germany. But we think that "superficial taxonomic impediment" (species are so poorly and unreliably named, they will need to be redescribed before they can be used) (Meier et al. 2022) is better applicable in this case. Thus, we can conclude that two sequences (MZ657748 and MZ658877) belong to well-known species that are absent in databases, or they can be considered as distinct species. A similar case was with Japanese *Ch. nipponensis*. At first, Yamamoto (2010, cited by Kondo et al. 2016) proposed dividing the "highland" and "lowland" populations of *Ch. nipponensis* by morphology, then Kondo et al. (2016) revealed the genetic distances between them at 9.1%, and confirmed the presence of two separate species.

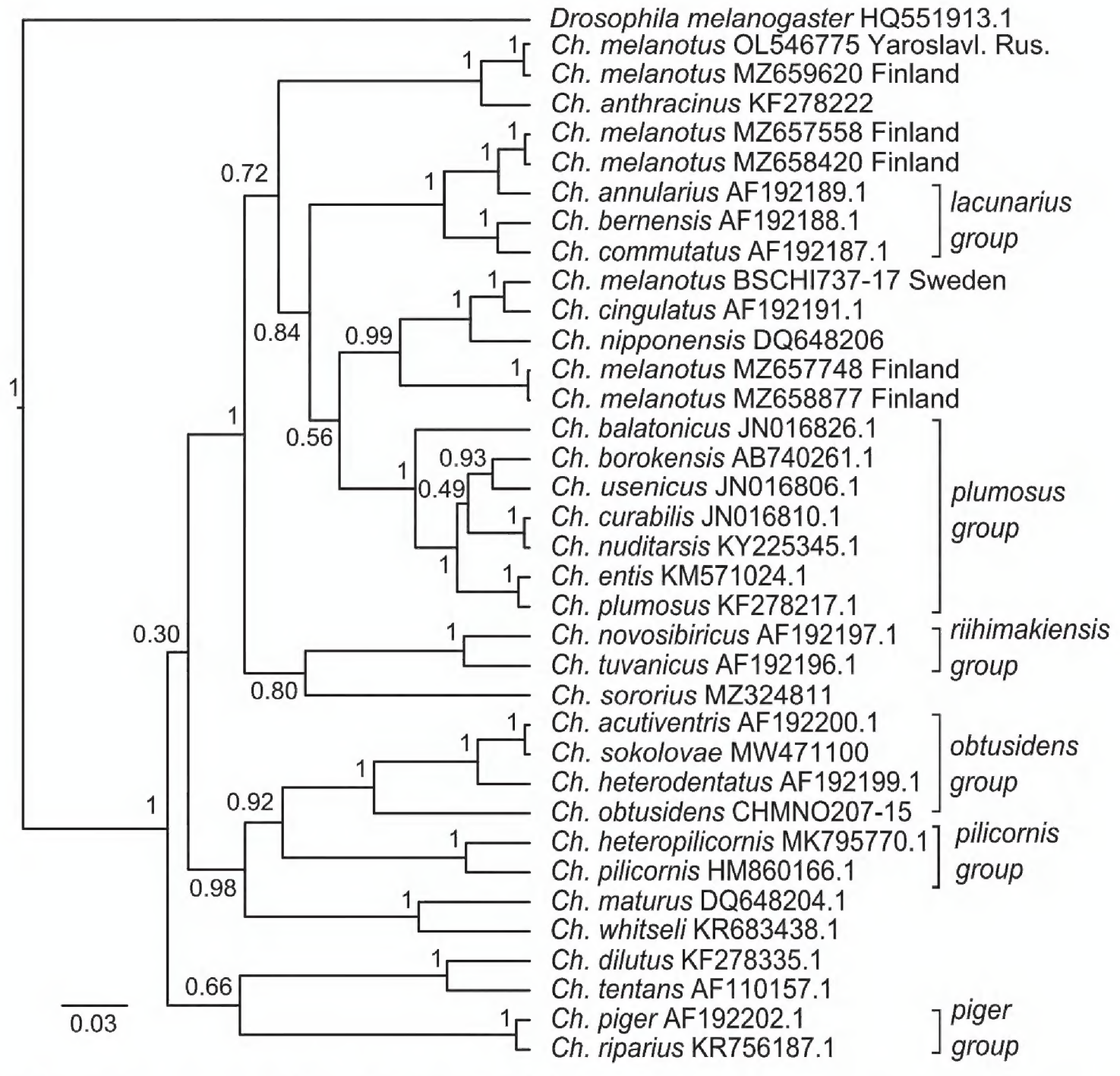

Figure 2. Bayesian tree of the analyzed samples of *Chironomus* spp. inferred from COI sequences. Species name, GenBank accession numbers and group name are shown to the right of the branches. Support values are given if they exceed 0.3. The numbers at the nodes indicate posterior probabilities.

Conclusions

On the example of *Ch. melanotus*, we confirmed that in *Chironomus* species identification we must use all available comprehensive approaches, involving morphological, cytogenetic and molecular genetic studies (mitochondrial and nuclear genes) (DeSalle et al. 2005; Ekrem et al. 2007; Proulx et al. 2013; Kondo et al. 2016).

At least four species of *Chironomus* could be in the databases under the name "*Ch. melanotus*" from Finland and Sweden. This suggests that at the present stage of the collection of genetic data, it is impossible to trust only a computer algorithm. We agree with Zamani et al. (2022) that the use of DNA-based analyses for an initial sorting of new and known species is extremely useful as a first step, which significantly narrows the range of search before precise species identification. Nevertheless, despite the difficulties, the species identification of *Chironomus* greatly enriches our understanding of ecosystem functioning because this is an important part of it.

Acknowledgements

The work was realized according to the Russia state projects 121050500046-8 and 121051100099-5. The authors are grateful to B.A. Levin, A.A. Bobrov (IBIW RAS) and M.Kh. Karmokov (IEMT RAS) for their help and consultations during all stages of the investigation and manuscript preparation; P. Michailova (IBER BAS), J. Martin (The University of Melbourne, Australia) and V.G. Kuznetsova (ZIN RAS) for the opportunity to see the work in a new light; D.D. Pavlov (IBIW RAS) for the linguistic corrections of the text.

References

Bolshakov VV, Prokin AA (2021) Karyotype and COI sequences of *Chironomus sokolovae* Istomina, Kiknadze et Siirin, 1999 (Diptera, Chironomidae) from the bay of Orkhon River, Mongolia. Comparative Cytogenetics 15(2): 149–157. https://doi.org/10.3897/CompCytogen.v15.i2.66549

Degelmann A, Roye HD, Hollenberg CP (1979) The organization of the ribosomal RNA genes of *Chironomus tentans* and some closely related species. Chromosoma 71(3): 263–281. https://doi.org/10.1007/BF00287136

DeSalle R, Egan MG, Siddall M (2005) The unholy trinity: taxonomy, species delimitation and DNA barcoding. Philosophical Transactions of the Royal Society B: Biological Sciences 360: 1905–1916. https://doi.org/10.1098/rstb.2005.1722

Dyomin SYu (1989) Variability of the degree of condensation of polytene chromosomes in the cells of different organs of *Chironomus plumosus* larvae from nature. PhD Thesis, Institute of Cytology of the USSR Academy of Sciences, Leningrad, 25 pp. [In Russian]

Ekrem T, Willassen E, Stur E (2007) A comprehensive DNA sequence library is essential for identification with DNA barcodes. Molecular Phylogenetics and Evolution 43(2): 530–542. https://doi.org/10.1016/j.ympev.2006.11.021

Fjellheim A, Raddum GG (1996) Weir building in a regulated west Norwegian river: Long-term dynamics of invertebrates and fish. Regulated Rivers: Research and Management 12(4–5): 501–508. https://doi.org/10.1002/(sici)1099-1646(199607)12:4/5<501::aid-rrr414>3.0.co;2-f

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294–299.

Gadawski P, Montagna M, Rossaro B, Giłka W, Pešić V, Grabowski M, Magoga G (2022) DNA barcoding of Chironomidae from the Lake Skadar region: Reference library and a comparative analysis of the European fauna. Diversity and Distributions: 1–20. https://doi.org/10.1111/ddi.13504

Grebenjuk LP (1994) Qualitative and quantitative composition of chironomids of the Latka River, a small tributary of the Rybinsk Reservoir. Biologiya vnutrennikh vod: Informatsionnyi byulleten 96: 40–46. [In Russian]

Grebenjuk LP, Tomilina II (2014) Morphological deformations of hard-chitinized mouthpart structures in larvae of the genus *Chironomus* (Diptera, Chironomidae) as the index of organic pollution in freshwater ecosystems. Inland Water Biology 7(3): 273–285. https://doi.org/10.1134/S1995082914030092

Guryev V, Makarevitch I, Blinov A, Martin J (2001) Phylogeny of the genus *Chironomus* (Diptera) inferred from DNA sequences of mitochondrial Cytochrome b and Cytochrome oxidase I. Molecular Phylogenetics and Evolution 19: 9–21. https://doi.org/10.1006/mpev.2001.0898

Hebert PDN, Ratnasingham S, Zakharov EV, Telfer AC, Levesque-Beaudin V, Milton MA, Pedersen S, Jannetta P, DeWaard JR (2016) Counting animal species with DNA barcodes: Canadian insects. Philosophical Transactions of the Royal Society B: Biological Sciences 371: 20150333. https://doi.org/10.1098/rstb.2015.0333

Hirvenoja M, Michailova P (1991) *Chironomus melanotus* Keyl and its taxonomical and ecological relations to *C. riihimakiensis* Wülker (Diptera, Chironomidae). Spixiana 14(3): 309–320.

Ilyinskaya NB (1983) The development of 4th instar larvae and diapause. In: Sokolova NYu (Ed.) *Chironomus plumosus* L. (Diptera, Chironomidae). Systematics, Morphology, Ecology, Production. Nauka publishers, Moscow, 167–188. [In Russian]

Int Panis L, Kiknadze II, Bervoets L, Aimanova K (1994) Karyological identification of some species of the genus *Chironomus* Meigen 1803 from Belgium. Bulletin & Annales de la Société Royale d'Entomologie de Belgique 130: 135–142.

Jabłońska-Barna I, Szarek-Gwiazda E, Michailova P (2013) Environmental agents in Lake Łuknajno (Poland) affecting the genome of *Chironomus melanotus* Keyl, 1961 (Diptera, Chironomidae) - A new species of Polish fauna. Oceanological and Hydrobiological Studies 42(2): 164–172. https://doi.org/10.2478/s13545-013-0070-2

Kang HJ, Baek MJ, Kang JH, Bae YJ (2022) Diversity and DNA Barcode Analysis of Chironomids (Diptera: Chironomidae) from Large Rivers in South Korea. Insects 13: 1–14. https://doi.org/10.3390/insects13040346

Karmokov MK (2019) Karyotype characteristics, chromosomal polymorphism and gene COI sequences of *Chironomus heteropilicornis* Wülker, 1996 (Diptera, Chironomidae) from the South Caucasus. Comparative Cytogenetics 13(4): 339–357. https://doi.org/10.3897/CompCytogen.v13i4.35572

Keyl H-G (1961) Die cytologische Diagnostik der Chironomiden III. Diagnose yon *Chironomus parathummi* n. sp. und Ergfinzungen zur Bestimmungstabelle. Archiv für Hydrobiologie 58: 1–6.

Keyl H-G (1962) Chromosomenevolution bei *Chironomus*. II. Chromosomenumbauten und phylogenetische Beziehungen der Arten. Chromosoma 13: 464–514. https://doi.org/10.1007/BF00327342

Kiknadze II, Shilova AI, Kerkis IE, Shobanov NA, Zelentsov NI, Grebenyuk LP, Istomina AG, Prasolov VA (1991) Karyoypes and larval morphology in tribe Chironomini. Atlas. Nauka Publish, Novosibirsk, 115 pp. [In Russian]

Kiknadze II, Butler MG, Gunderina LI, Istomina AG, Gusev VD, Nemytikova LA (2010) Chromosomal Evolution of Nearctic and Palearctic *Chironomus* Species (Diptera: Chironomidae). Proceedings of the XV international Symposium on Chironomidae, Chironomidae Research Group, University of Minnesota, Saint Paul, Minnesota, 203–221.

Kiknadze II, Istomina AG (2000) Karyotypes and Chromosomal Polymorphisms in Siberian Chironomids (Diptera, Chironomidae). Sibirskiy ekologicheskiy zhurnal 4: 445–460. [In Russian]

Kiknadze II, Istomina AV, Golygina VV, Gunderina LI (2016) Karyotypes of Palearctic and Holarctic species of the genus *Chironomus* [Electronic resource]. Russian Academy of Sciences, Siberian Branch, Federal Research Center Institute of Cytology and Genetics. Novosibirsk, 489 pp.

Kondo NI, Ueno R, Ohbayashi K, Golygina VV, Takamura K (2016) DNA barcoding supports reclassification of Japanese *Chironomus* species (Diptera: Chironomidae). Entomological Science 19(4): 337–350. https://doi.org/10.1111/ens.12212

Meier R, Blaimer BB, Buenaventura E, Hartop E, Rintelen T, Srivathsan A, Yeo D (2022) A re-analysis of the data in Sharkey et al.'s (2021) minimalist revision reveals that BINs do not deserve names, but BOLD Systems needs a stronger commitment to open science. Cladistics 38: 264–275. https://doi.org/10.1111/cla.12489

Morinière J, Balke M, Doczkal D, Geiger MF, Hardulak LA, Haszprunar G, Hausmann A, Hendrich L, Regalado L, Rulik B, Schmidt S, Wägele J-W, Hebert PDN (2019) A DNA barcode library for 5,200 German flies and midges (Insecta: Diptera) and its implications for metabarcoding-based biomonitoring. Molecular Ecology Resources 19(4): 900–928. https://doi.org/10.1111/1755-0998.13022

Proulx I, Martin J, Carew M, Hare L (2013) Using various lines of evidence to identify *Chironomus* species (Diptera: Chironomidae) in eastern Canadian lakes. Zootaxa 3741: 401–458. https://doi.org/10.11646/zootaxa.3741.4.1

Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Hohna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61: 539–542. https://doi.org/10.1093/sysbio/sys029

Roslin T, Somervuo P, Pentinsaari M, Hebert PDN, Agda J, Ahlroth P, Anttonen P, Aspi J, Blagoev G, Blanco S, Chan D, Clayhills T, deWaard J, deWaard S, Elliot T, Elo R, Haapala S, Helve E, Ilmonen J, Hirvonen P, Ho C, Itämies J, IvanovV, Jakovlev J, Juslén A, Jussila R, Kahanpää J, Kaila L, Kaitila J-P, Kakko A, Kakko I, Karhu A, Karjalainen S, Kjaerandsen J, Koskinen J, Laasonen EM, Laasonen L, Laine E, Lampila P, Levesque-Beaudin V, Lu L, Lähteenaro M, Majuri P, Malmberg S, Manjunath R, Martikainen P, Mattila J, McKeown J, Metsälä P, Miklasevskaja M, Miller M, Miskie R, Muinonen A, Mukkala V-M, Naik S, Nikolova N, Nupponen K, Ovaskainen O, Österblad I, Paasivirta L, Pajunen T, Parkko P, Paukkunen J, Penttinen R, Perez K, Pohjoismäki J, Prosser S, Raekunnas M, Rahulan M, Rannisto M, Ratnasingham S, Raukko P, Rinne A, Rintala T, Romo SM, Salmela J, Salokannel J, Savolainen R, Schulman L, Sihvonen P, Soliman D,Sones J, Steinke C, Ståhls G,Tabell J, Tiusanen M,Várkonyi G, Vesterinen EJ, Viitanen E, Vikberg V, Viitasaari M, Vilen J, Warne C, Wei C, Winqvist K, Zakharov E, Mutanen M (2022) A molecular-based identification resource for the arthropods of Finland. Molecular Ecology Resources 22(2): 803–822. https://doi.org/10.1111/1755-0998.13510

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30: 2725–2729. https://doi.org/10.1093/molbev/mst197

Zamani A, Fric ZF, Gante HF, Hopkins T, Orfinger AB, Scherz MD, Bartoňová AS, Pos DD (2022) DNA barcodes on their own are not enough to describe a species. Systematic Entomology 1–5. https://doi.org/10.1111/syen.12538

Wülker WF (1973) Revision der Gattung *Chironomus* Meig. III. Europaische arten des thummi-Komplexes. Archiv für Hydrobiologie 72: 356–374.

Wülker WF, Butler MG (1983) Karyosystematics and morphology of Northern *Chironomus* (Diptera: Chironomidae): Freshwater species with larvae of the salinarius-type. Entomologica Scandinavica 14: 121–136. https://doi.org/10.1163/187631283X00010

Wülker W, Martin J (2000) Northernmost *Chironomus* karyotypes (Insecta, Diptera, Chironomidae). Spixiana 23: 151–156.

ORCID

Viktor V. Bolshakov https://orcid.org/0000-0002-8028-3818
Ekaterina A. Movergoz https://orcid.org/0000-0002-3677-6631